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14. ABSTRACT

Purpose: To test the hypothesis that there are growth factors/cytokines released to promote healingof the wound created by the resection of a primary breast cancer which have the unintended consequence of enabling the growth of micrometastatic foci present at the time of operation. Objectives: This study was designed as a feasibility study to test whether it is technically possible to reliably assay changes in the low molecular weight serum proteome. Major Findings: Human xenograft breast tumors were established in 9 of 12 nude mice. Blood sampleswere obtained from the mice immediately prior to extirpation of the primary breast cancer and then again 24 hours, 48 hours and 7 day post-operatively. In the analysis of the serum of 5 of the mice there were 8685 peptides quantified resulting in 5949 proteins. Of these 5949 proteins 155 were identified with high confidence and 11 proteins had significant changes among the time points as a function of time pre or post-op. One of these proteins is a splice variant of the Leukemia Inhibitory Factor. We have cloned three forms of this receptor, the full length, secretory and membrane forms.

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INTRODUCTION

There are numerous accounts in the literature dating back to the late 1940s that report that the removal of a primary cancer results in the growth of metastases or of a second tumor transplant[1-7] Mouse models reveal that there is a substance(s) released into the blood stream following primary tumor removal which stimulates the growth of secondary tumors[8]. Breast cancer patient recurrence and mortality data suggest that there is a perturbation of the growth of metastatic deposits that is at least coincident with, if not, consequent to operative intervention[9-11]. Wound drainage fluid as well as serum collected 24 hours after either lumpectomy or mastectomy results in an increase in proliferation in all breast cancer cell lines tested in an *in vitro* assay[12]. We hypothesized that growth factors released to promote healing of the wound created by resection of the primary tumor, result in the expression of other effectors, e.g., growth factors and cytokines, which stimulate the proliferation of cancer cells present in micrometastatic foci. This, in turn, results in excess mortality directly attributable to the operation and is a phenomenon which can be prevented. The objective of the research funded by this Concept Award from the Department of Defense was to determine the feasibility of identifying and quantifying the proteins/peptides released, synthesized or eliminated in response to operative therapy.

BODY

A human breast cancer xenograft mouse model developed by Dr. Harikrishna Nakshatri at Indiana University was ideally suited for this study because the breast cancers that develop do not metastasize to the lungs if the tumors are left undisturbed. However, if the breast cancers are surgically removed, 60-70% of the mice develop lung metastases (personal communication). 12 mice nu/nu were anesthetized and then inoculated with TMD-231 cells. Palpable tumors developed in 8 of 11 mice. One mouse was found dead in her cage of unknown cause. Tumors were resected after 8 weeks. One mouse expired during the extirpation of the tumor due to exsanguination. A blood samples (40 μl) were taken immediately prior to tumor resection and then again 24, 48 and 120 hours after removal of the tumor. Blood was obtained from the facial vein/artery, alternating sides with each subsequent bleed. Blood was collected in Sarstedt Microvette 100z capillary tubes. Specimens were centrifuged 1 minute, 2000 RPM (Biofuge fresco, Hearaeus), 4˚C to transfer the blood from the capillary tube to the polypropylene tube to which it was connected. The capillary tubes were then removed and the polypropylene tubes centrifuged 5 minutes, 12,000 RPM (Biofuge fresco, Hearaeus), 4˚C to separate the serum. The serum was pipetted into cryovials which were placed into an isopropyl alcohol freezing container which was placed into a -70˚C freezer. After all serum samples had been obtained and were frozen, the serum was transferred for storage in liquid nitrogen until transfer to the Proteomics Core Laboratory. Of the 12 mice, sera from 7 were available for analysis.

Sample preparation for proteomics:

Albumin and IgG were depleted from the mouse serum samples by Montage™ (Millipore) and Protein G (Amersham) spin columns. The resulting depleted serum samples were denatured by 8M urea, reduced by triethylphosphine, alkylated by iodoethanol, and digested by trypsin[13]. This allows all steps to be carried out in one tube without washing or filtering steps.

Protein Identification:

Proteins were identified using two different proteomic methodologies. The first method is called Multi-dimensional Protein Identification Technology (MudPIT). For MudPIT analysis intact serum proteins were proteolytically digested to form a mixture of peptides and were analyzed directly by multidimensional liquid-chromatography, specifically a strong cation exchange column followed by a C-18 reverse phase column, and tandem mass spectrometry. An off-line micro fraction collector was used to collect fractions as they eluted from the cation exchanger. This enabled the use of a continuous salt gradient in the first separation dimension significantly increasing the number of proteins identified in comparison to stepwise elution. Acquired peptide fragmentation spectra were then correlated with predicted amino acid sequences in translated genomic databases using the SEQUEST™ algorithm (Thermo-Finnigan). The advantage of MudPIT is that is enabled the identification of 25-30% more proteins when compared with one dimensional chromatography in a comparative study carried out in our Proteomics Core facility. Its disadvantage is that quantitation is not possible and therefore all data generated is qualitative.

The second method utilized was a label-free single dimension liquid chromatography/mass spectroscopy based quantitative protein analysis. This unique technology combines a proprietary sample preparation protocol[13], liquid chromatography/mass spectroscopy and data analysis tools. It increases the quantifiable protein dynamic range 4- to 5- fold as compared to gel based approaches. Tryptic peptides (~20 μg) were analyzed using a Thermo-Finnigan linear ion-trap mass spectrometer (LTQ) coupled with a HPLC system. A C18 reverse phase column (i.d.=1 mm, length=50 mm) was used to separate peptides with a flow rate of 50 μL/min. Peptides were eluted with a gradient from 5 to 45% acetonitrile developed over 120 min and data were collected in the triple-play mode. Triple play is a Thermo-Finnigan term, meaning: 1) parent ion scan (MS, peptide detection); 2) zoom scan (charge state determination); and 3) MS/MS scan (peptide sequence determination). This system and method can detect at least 1-2 peptides per MS/MS scan. The resulting MS/MS data were applied for database search using SEQUEST™ algorithm (Thermo-Finnigan). Various data processing filters were used to assure that only peptides with the *XCorr* score above 2.0 for singly charged, 2.5 for doubly charged, and 3.8 for triply charged peptides, were analyzed for protein identity. *XCorr* is a cross correlation provided by SEQUEST™ to measure the quality of the peptide identification [the bigger the better]. We were able to obtain quantitative data for up to twenty proteins from each parent ion scan. Using proprietary software[14] and statistical analysis tools, confirmed differentially expressed proteins were identified and the direction of change (up- or down-regulation) was also determined. Also an approximate fold change was calculated, but this is primarily used to determine the significance of the change and not the absolute level of the change (See section below). All data processing were carried out on a Linux cluster using highly parallel processing and proprietary data qualification and filtering software licensed from Eli Lilly and Company (Indianapolis, IN). Data were then statistically analyzed using multiple proprietary and commercial tools including SAS (See section below).

Protein Quantification: Protein quantification was carried out using the non-gel based and label-free proprietary protein quantification technology that the Core lab has licensed from Eli Lilly and Company. Briefly, once the raw files were acquired from the LTQ, all total ion chromatogram (TIC) were aligned by retention time. Each aligned peak should match parent ion, charge state, daughter ions (MS/MS data) and retention time (within 1 minute window). If any of these parameters were not matched, the peak was thrown out from the quantification. The integral volume under the curve from individually aligned peaks was measured, normalized, and compared for their relative abundance. An example of this quantification process is shown in Fig. 1.

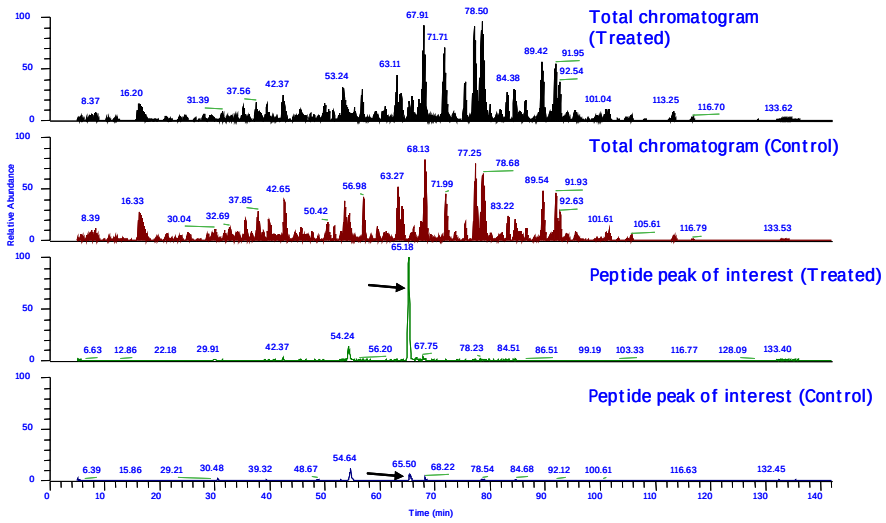

Figure 1. Peptide (protein) quantification by LC/MS.

Qualitative Analysis: Although historically 60-70% of mice using this model of breast cancer develop lung metastasis, only one mouse in this study developed gross metastatic disease. Therefore, the mice were analyzed as follows:
Sera from the mouse with gross metastatic disease and from one of the mice without metastatic disease were analyzed using MudPIT. As this project had been designed as a feasibility study, MudPIT was chosen as the initial proteomic method as it would yield the maximum number of protein identifications. This analysis cost $27,000 and therefore was limited to the sera from these two mice.

Table 1. Results of MudPIT

sample #	peptides	proteins	highly confident ID
Mouse 5/Bleed 1	23868	3265	367
Mouse 5/Bleed 2	15402	3250	426
Mouse 5/Bleed 3	13661	2796	389
Mouse 5/Bleed 4	13184	2159	248
Mouse 10/Bleed 1	14878	3840	503
Mouse 10/Bleed 2	15349	3765	583
Mouse 10/Bleed 3	16668	3434	498
Mouse 10/Bleed 4	15239	3948	502

In order to identify a protein with “high confidence” two or more unique peptides must be identified.

Quantitative Analysis:

The same sera as was used for the above qualitative analysis were then analyzed utilizing the label-free single dimension liquid chromatography/mass spectroscopy based quantitative protein analysis. There were 5109 peptides quantified resulting in 3458 proteins. Of these 3458 proteins there were 102 proteins identified with high confidence (Priority 1). Of the 102 Priority 1 proteins there were 57 that had significant changes as a function of time with respect to the operation. The significance threshold was set to control the False Discovery Rate (FDR) at less than 5%. A False Discovery is a protein declared significant when it isn't. The replicate median % Coefficient of Variation (CV) for the Priority 1 proteins was 4.61%. There were also 1271 proteins that had significant changes among the 3356 proteins that were less confidently identified (Priorities 2-6). The considerations for assigning proteins to the different priority groups are discussed in detail in the statistical summaries in the appendices. Based upon these promising preliminary results, the sera from the remaining 5 mice were analyzed for quantitative differences.

Rank	Priority	Annotation	Max Fold Change	Significant Change
1	1	similar_to_KIAA1336_protein	1.25728	YES
2	1	Apolipoprotein_A-I_precursor	1.28167	YES
3	1	Zinc_finger_Y-chromosomal_protein_1	1.26396	YES
4	1	_Es1_protein_[Mus_musculus]	1.32484	YES
5	1	Serum_amyloid_A-2_protein_precursor_[Contains:_Amyloid_protein_A_(Amyloid_fibril_protein_AA)]	1.9204	YES
6	1	Apolipoprotein_C-II_precursor	1.23252	YES
7	3	Splice_isoform_1_of_P42703_Leukemia_inhibitory_factor_receptor_precursor	2.14655	YES
8	3	B26300_alpha-1-acid_glycoprotein_(clone_pMAGP3)_-_mouse_(fragment)	1.38323	YES
9	3	Splice_isoform_3_of_O08715_A_kinase_anchor_protein_1,_mitochondrial_precursor	1.58822	YES
10	3	COP9_complex_subunit_6_(COP9	1.58814	YES
11	3	1600031J20Rik_protein	2.73699	YES
12	1	Serum_amyloid_P-component_precursor	1.59702	NO
13	1	Calcium-sensitive_chloride_conductance_protein-1	1.24509	NO
14	1	sex-limited_protein	1.21796	NO
15	1	Transthyretin_precursor	1.52952	NO
16	1	Serotransferrin_precursor	1.17545	NO
17	1	Alpha-1-acid_glycoprotein_1_precursor	1.43212	NO
18	1	Splice_isoform_HMW_of_O08677_Kininogen_precursor_[Contains:_Bradykinin]	1.17502	NO
19	1	Hypothetical_protein	1.1552	NO
20	1	Corticosteroid-binding_globulin_precursor	1.29848	NO
21	1	Alpha-1-acid_glycoprotein_2_precursor	1.38185	NO
22	1	Afamin_precursor	1.09414	NO
23	1	Splice_isoform_1_of_Q01705_Neurogenic_locus_notch_homolog_protein_1_precursor	1.24577	NO
24	1	Serum_amyloid_A-1_protein_precursor	1.87059	NO
25	1	hypothetical_protein_XP_358204	1.18165	NO

Table 2. Sample Results of Quantitative Analysis

For the remaining 5 mice there were 8685 peptides quantified resulting in 5949 proteins. Of these 5949 proteins there were 155 proteins identified with high confidence (Priority 1). Of the 155 Priority 1 proteins there were 6 that had significant changes among groups (time points). The replicate median % Coefficient of Variation (CV) for the Priority 1 proteins was 6.18% and the combined replicate and sample median %CV was 10.03%. The %CV is the Standard Deviation divided by the Mean on a % scale. There were 5 proteins that had significant changes among the 5794 proteins that were less confidently identified (Priorities 2-6). A sample of the data is presented in table 2.

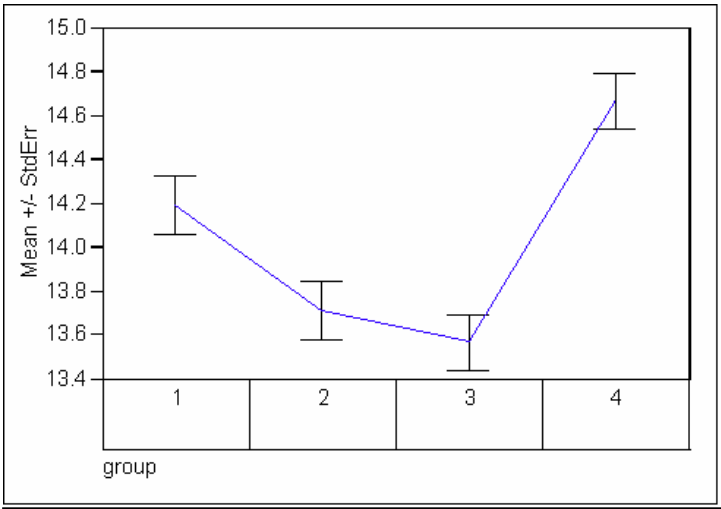

Figure 2. Change of Leukemia Inhibitory Factor Receptor as a Function of Time Pre- and Post-tumor Extirpation

One of the proteins identified with a significant change over the four time points is a splice form of the leukemia inhibitory factor receptor (LIFR). LIF, the ligand of this receptor, has been shown to inhibit the growth of MCF-7 cells[15], and to inhibit the proliferation of non-malignant breast epithelial cells with a reduction in S phase and an increase in cells in G_0/G_1[16]. Data published by Gunduz *et al* indicates that the increase in growth of secondary or metastatic lesions is the result of the recruitment of cells out of G_0[1]. We also note that stem cells reside in G_0. Data from our study shows that LIFR decreases immediately post-operatively with its nadir at 48 hours as shown in Figure 3 below. From this we can hypothesis that the decrease in LIFR may release a brake on proliferation which allows the quiescent micrometastatic cells (stem cells?) to re-enter the cell cycle. We were fortunate to recruit a postdoctoral fellow, Mi Ran Choi, Ph.D. in April of this year. Dr. Choi has assumed responsibility for this project. She has cloned 3 forms of the LIFR: full length, secretory and membrane bound, from a choriocarcinoma library. These clones, at the present time, are being sequenced.

KEY RESEARCH ACCOMPLISHMENTS

- Using two-dimensional column chromatography and mass spectroscopy (MudPIT) we were able to identify hundreds of serum proteins at each time point prior to and following tumor extirpation.
- Using a label-free single dimension liquid chromatography/mass spectroscopy based quantitative protein analysis we were able to identify 5949 proteins of which 155 were identified with a high confidence. 11 of the 5949 proteins had statistically significant changes among the time points relative to tumor extirpation.

REPORTABLE OUTCOMES

- Articles regarding this research were published in the Wall Street Journal September 13, 2005 and Baltimore Sun May 26, 2006.
- Abstract submitted for presentation at the 28th Annual San Antonio Breast Cancer Symposium. Abstract appended to August 2005 report.
- Abstract submitted for presentation at the Annual Meeting of the Society of Surgical Oncology, March 2006.
- Abstract submitted for presentation at the 29th Annual San Antonio Breast Cancer Symposium.

- Collaborative relationship established with Professor Michael Baum, University College, London. Professor Baum has published[17] and spoken extensively (William McGuire lecture presented at the 25th Annual San Antonio Breast Cancer Symposium) on the subject of operative therapy and its hypothesized effects on vascularization of micrometastses.

CONCLUSIONS

This proposal was designed as a feasibility study, that is, to determine if it is technically possible to reliably assay changes in the low molecular weight serum proteome. We have demonstrated that this is technically possible in two different senses:

- 1.) We have been able to identify hundreds of proteins with high confidence in regard to the reliability of the identification and
- 2.) The technical variation, i.e., the variation of the measurements, was shown to be relatively small with Coefficients of Variation between injections into the mass spectrometer of 4-6%.

We plan to repeat the experiments using a larger cohort of mice so that the number of mice developing metastases is great enough to make the results statistically meaningful. The ultimate goal of this research in therapeutic. If we can demonstrate that certain proteins/peptides present in the serum following removal of a primary breast cancer have a direct effect on the growth of metastatic lesions, these proteins/peptides will become potential targets for perioperative therapy. The actions of these proteins may be able to be blocked by administering antibodies and/or small molecule drugs.

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